Assessing the Utility of AFLP for Determining Mutational Changes in Coral

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Introduction

Degradation of the world's coral reefs is one of the most vivid examples of the effects of global environmental damage of marine ecosystems. The major stressors responsible for coral reef decline have been attributed to coastal urban and industrial development, agricultural activity, sedimentation, overharvesting, marine pollution, disease and climate change (Walker & Ormond 1982; Bryant *et al*. 1998; Risk 1999; Turgeon *et al*. 2002; Bellwood *et al.* 2004). Reef species experiencing persistent environmental disturbances (e.g., coastal development and land-based pollution) may exhibit acute mortality leading to a seemingly rapid loss of coral-reef diversity and abundance, but may also display non-acute, sub-lethal effects. These effects are often present as increased incidence of disease, altered growth and regeneration rates, reduced reproductive effort and reduced recruitment, which can ultimately result in a cascading effect of ecosystem deterioration (Richmond 1993; Hoegh-Guldberg 1999; Nystrom *et al*., 2000; Knowlton 2001; Porter & Tougas 2001; CRMP 2001; Patterson *et al.* 2002).

The ecological consequences to coral and coral reefs of exposures to increasing amounts and types of pollutants are difficult or impossible to assess in short-term (less than five years) studies. One of the most informative endpoints to measure the risk of these exposures is reproductive fitness, though this too is logistically challenging, time-consuming and expensive. However, indirect measures of reproductive fitness can be made through carefully selected proxies that are well supported and accepted in the research community, and which reflect specific aspects of reproductive physiological condition. Markers that reflect conditions of genomic integrity provide a group of such proxies (Moore *et al*. 2004; Ricketts *et al*. 2004; Jha 2008)

Genotoxic compounds can act directly through the accumulation of deleterious mutations or indirectly by affecting the organism's physiology or environment, but in either instance they often affect survival and/or fecundity (De Wolf *et al*. 2004). Studying the direct effects of contaminants on DNA structure and function are important because DNA is the foundation of reproduction and inheritance, and changes in its structure or function often lead to population level changes, i.e., affecting population structure or demographics (Theodorakis 2001). There are numerous techniques for assessing structural or functional changes in DNA. These include markers such as those measuring DNA damage (e.g., DNA abasic sites, COMET assay), DNA adducts (e.g., 8-oxo-dG) or mutations (e.g., RAPDs, SNPs, RFLPs, SSCPs).

Cells are equipped with DNA repair systems and can combat the effects of genotoxic compounds, thus information from a single evaluation of DNA integrity provides only a snapshot of DNA damage to that organism. These assays cannot determine the net effect of such exposures. To address this gap in information, an assay that could determine accumulation of mutations over time would be a valuable tool for determining the risk of pollutant exposures to coral health. The AFLP technique was selected for evaluation because large portions of the genome could be sampled and it does not require *a priori* DNA sequence information. Two desirable features of this assay are that it is applicable to any species and is relatively low cost (Amar *et al*. 2008). Furthermore, since it is based on amplification of genomic restriction fragments, the technique is robust and reliable due to the assay's stringent conditions (Vos *et al*. 1995).

Rationale

The AFLP assay is a DNA fingerprinting technique, which involves electrophoresis of DNA fragments from an organism or cell sample in a gel matrix, to generate a unique banding profile. Most current DNA fingerprinting techniques use PCR to generate the fragments (e.g., RAPDs, DGGE, AP-PCR). The major disadvantage of these fingerprinting techniques is that they are sensitive to DNA quality, reaction conditions and reaction temperature profiles (Vos *et al*. 1995). Because of these issues, several of these techniques have come under significant criticism (Atienzar & Jha 2006).

The AFLP technique was originally developed by Vos *et al*. (1995) for genotyping individuals and has also been used for population structure analyses (Amar *et al*. 2008), and mutation rate determinations (Kropf *et al*. 2009). This technique overcomes weaknesses in other DNA fingerprinting assays by combining the specificity of DNA restriction enzymes with amplification of fragments by PCR.

There are four key steps to the method: 1) extraction and restriction digestion of genomic DNA, 2) ligation of adapters to the ends of the restriction fragments, 3) selective amplification of the modified fragments, and 4) electrophoresis of the resulting products. The genomic DNA of samples is digested with two different restriction enzymes, one with a 4-base and the other with a 6-base recognition sequence. Pre-selective PCR primers with two-base overhangs are matched with adapters related to the restriction enzyme recognition sites to selectively reduce the number of DNA fragments. Amplification reactions are performed using primers with three-base overhangs and labeled such that only fragments containing the restrictions sites used initially, will be detected. PCR products are then analyzed by gel or capillary electrophoresis. Fragments are binned and then analyzed for fingerprint similarity with other samples.

The objective of the two-enzyme restriction cut is to generate DNA fragments of optimal size for amplification and of a size easily separated on polyacrylamide denaturing gels. This double cut strategy also reduces the number of fragments that will amplify to only a subset of the restriction fragments, using PCR primers for the adapters. If needed, this approach provides a means to selectively label one strand of the PCR products to prevent mobility differences between double strands. In

addition, it allows optimization of fragment numbers while maintaining stringent assay conditions, not available in other DNA fingerprinting techniques (Vos *et al*. 1995).

Strategy

The AFLP assay has been commercialized by several companies (e.g., Life Technologies, Licor, Beckman Coulter, Applied Biosystems) into a kit format for various platforms (e.g., capillary electrophoresis, gel-based systems). The Applied Biosystem kits (Amplification Core Mix Module, Cat. # 402005 and AFLP Ligation and Preselective Amplification kit, Cat. # 402004) were used as the basis for this evaluation. It should be noted that coral tissues include symbiotic algae and a unique surface microbial community that can be sources of non-coral DNA which could confound results (Amar *et al*. 2008). To minimize these sources of possible artifacts, a strategy was developed that uses the standard AFLP protocol to identify candidate coral genomic fragments and validate them by DNA cloning and sequencing. Validated coral genomic fragments are used to design an assay for assessing accumulation of mutations in corals exposed to potentially genotoxic compounds. The accumulation of mutations is then assessed on coral-specific fragments from field samples collected using a repeated measures design (i.e., resampling the same individual over time). Direct sequencing or RFLP-type analysis is used to quantify mutations. Finally a practical assay needs to be amenable to high-throughput analysis.

Standard AFLP Protocol

DNA isolation - Samples of *Porites lobata* previously collected from reference and impacted field sites were used as source materials for this evaluation. DNA was isolated from frozen cryomilled samples using the GetPure DNA kit (Dojindo Molecular Technologies), with a minor modification of the addition of polyvinylpolypyrrolidone (PVPP) to bind polyphenolic compounds inherent in coral tissues (May & Woodley In Press; also http://cdhc.noaa.gov/_docs/Virtual%20Chemiluminescent%20DNA%20AP%20Site%20Assay_formatted_11-15-11.pdf). Briefly, ~50 mg of frozen cryomilled coral tissue was placed into a 1.5 ml Eppendorf tube containing 400 µl of room temperature lysis buffer from the kit and ~15 mg of PVPP. The remaining steps were conducted according to the manufacturer's instructions. The DNA yield was determined using the Quant-iT kit (Invitrogen, Life Technologies) and its integrity by agarose gel electrophoresis.

Genomic DNA restriction digests - High molecular weight DNA (~100 ng) was double-digested with 500 units of *Eco*RI (50,000 units/ml; New England Biolabs), and 100 units of *Mse*I (10,000 units/ml; New England Biolabs), overnight at 37°C. Complete digestion of the genomic DNA was assessed by agarose gel electrophoresis.

Adapter ligation – Using the Applied Biosystems® AFLP kit (Life Technologies, Grand Island, NY) components, *Eco*RI and *Mse*I adaptors were ligated onto the fragmented DNA by incubating overnight at room temperature with T4 DNA ligase. The adapters consist of a core sequence, an enzyme specific sequence, and a selective extension with usually three selective nucleotides (Vos *et al*. 1995).

Preselective PCR amplification – The first amplification used primers corresponding to each end of the modified restriction

fragments plus one selective nucleotide to provide sufficient material for subsequent selective PCR. The template of genomic DNA fragments with modified adaptors was PCR amplified using pre-selective primers from the kit with the following conditions: initial denaturation at 94°C for 2 min, twenty cycles of 94°C for 20 s, 56°C for 30 s and 72°C for 2 min, followed by a final extension of 60°C for 30 min and a hold at 4°C.

Selective PCR amplification - In a second amplification reaction, primers with selective nucleotides included at the 3' ends were used to amplify a subset of restriction sites, since only the restriction fragments having these specific nucleotides flanking the restriction site will match and be amplified. Eight pairs of selective primers (kit components) were evaluated using the

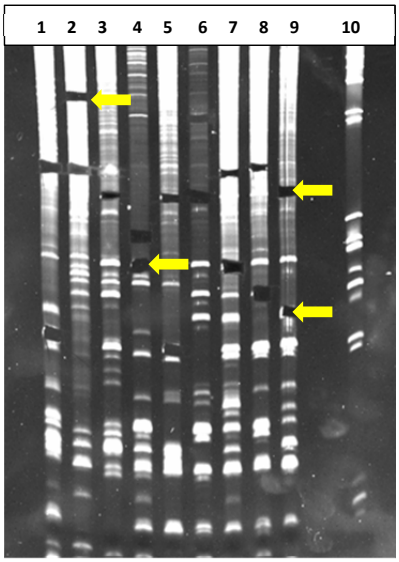

Figure 1 AFLP profiles from individual *Porites lobata* samples (lanes 1-9). Arrows indicate examples of the location where DNA fragments were cut from a 6% denaturing polyacrylamide gel for cloning. Lane 10 is a control.

test DNA for amplification to determine which pair provided optimal banding profiles. Reaction conditions for these tests were conducted according to the manufacturer's instructions, and included a touchdown annealing step from 66°C (-1 °C/cycle) for ten cycles followed by twenty cycles with a 56°C annealing temperature. Two primer pairs selected for further analysis were *Eco*RI-ACA/*Mse*I-CTA and *Eco*RI-ACA/*Mse*I-CAG.

Cloning of AFLP fragments-PCR products from selectively amplified AFLP DNA were subjected to denaturing polyacrylamide electrophoresis (6% polyacrylamide, 7 M urea) and bands were excised from each DNA sample, targeting those estimated to be ~1 kb (e.g., Fig. 1). Each DNA fragment was eluted from the excised gel in sterile deionized water at 4°C overnight and cloned into the pCR2.1 vector using a TOPO-TA cloning kit (Invitrogen).

Clones were evaluated for insert size by PCR amplification using primers from the multiple cloning sites of the vector and electrophoresis on a 0.8% agarose gel. All of the resulting inserts from the cloned fragments however were smaller than 500 bp. The fact that these fragments were smaller than anticipated is likely due to the denaturing gel electrophoresis conditions and also the standard AFLP protocol was designed for targets generally 1 kb or less. These small fragments were deemed unsuitable for determining mutation accumulation. It was concluded that for the intended application, the procedure required modification to obtain larger fragments.

Modified AFLP Protocol

Modification of AFLP protocol to increase fragment size – Three options were considered to increase the fragment sizes: 1) change the enzyme combinations in the initial genomic digest, 2) conduct blunt-end

digestions, or 3) digest the genomic DNA with *Eco*RI alone. Option three was selected for further evaluation because it was the most efficient while continuing to provide a means of selective amplification.

The AFLP protocol was followed essentially as described above except only one enzyme was used in the restriction digest, *Eco*RI. Accordingly, twice the amount of *Eco*RI adaptors were used and the *Mse*I adaptors were omitted from the ligation reaction. The pre-amplification products then were amplified with the *Eco*RI selective primer *Eco*RI-ACA only. Products were separated in a 6% denaturing polyacrylamide gel and bands cut from the gel and eluted as described above. Re-amplification of these bands again yielded fragments <600 bp.

With the goal of producing larger fragments, two new selective primer sets (EcoRI-ACA/EcoRI-AAG and EcoRI-AAG/EcoRI-AAC) were evaluated. Amplification products were separated on a 6% denaturing polyacrylamide gel that was run for 20 hr at 400V. The extended electrophoresis time and lower voltage as compared to previous runs were used to separate the largest fragments from the amplification products. A subset of bands was cut from this gel and eluted. The eluted DNA was re-amplified using the same primer sets and evaluated for fragment size and number on a 1% agarose gel (Fig. 2). These conditions yielded many large fragments of approximately 1 kb in length. These fragments were cloned and 96 clones were sequenced.

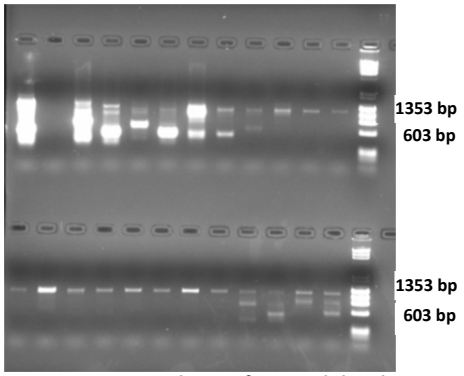

Figure 2 PCR products of excised bands separated in 0.8% agarose gel showing fragments >1 kb (lanes 1-12 top & bottom). Lane 13 top & bottom HaeIII digested ØX174 MW marker.

Following a Basic Local Alignment Search Tool (BLAST) (Altschul *et al*. 1990) analysis of the cloned DNA, seven target coral sequences were identified. Oligonucleotide primers were designed to target each of the seven putative coral sequences as shown in Table I. Primer pairs were tested against 50 field samples to determine whether individual fragments could be amplified from multiple colonies of a given species and thus meet a critical criterion. Test samples included *Porites lobata* from Hawaii: Maunalua Bay (6 subsites, 23 colonies), Ordnance Reef (2 subsites, 7 colonies), West Maui (2 subsites, 17 colonies), and La Perouse (1 subsite, 3 colonies). Additionally, the primers were tested on a cultured coral, *Pocillopora damicornis* (5 colonies) from the CCEHBR Coral Husbandry Facility, Charleston, SC.

Results

The modifications made to the standard AFLP protocol did yield larger sized AFLP fragments (Fig. 2). However, when AFLP fragments were cloned and sequenced, the BLAST results indicated that many of the clones were of bacterial origin (data not shown). This confirmed our suspicions that the standard AFLP method was not coral-specific in the banding profiles it generated and thus could not be used to determine mutational changes for coral. Thus, our strategy to isolate coral-specific genomic fragments was necessary to be able to

assess mutation frequency in coral, however to now use coral-specific genomic fragments would require designing a new assay platform.

In an effort to design a screening assay to detect increased mutations in corals exposed to potentially genotoxic pollutants, seven coral-specific genomic sequences >1kb that had been isolated from AFLP polyacrylamide gels, cloned and verified by DNA sequencing (gel not shown) were used to design PCR primers for testing individual specimens. PCR products of the correct target size indicated that the corresponding restriction sites (at the end of each fragment) were intact across individuals. All primer pairs except C315F/C316R successfully amplified the target coral sequences, but required predigestion of the genomic DNA with *Eco*RI for a successful amplification (Fig. 3 example of agrose gel screening). Oligonucleotide primers, C315F/C316R, failed to amplify any *P. lobata* samples, possibly due to inaccuracies in the sequence data, or *Eco*RI star activity during the template restriction digest. In some instances, only a few *P. lobata* samples failed to yield PCR products with certain primer pairs. The oligonucleotide primer combination that successfully amplified targets from the highest number of samples was C303/C304 (amplified 76.1 % of samples). Primer pair C313/C314 had the lowest rate of success with only 16.4 % of coral samples amplified. All other primer pairs amplified between 58.2- 69.1 % of samples.

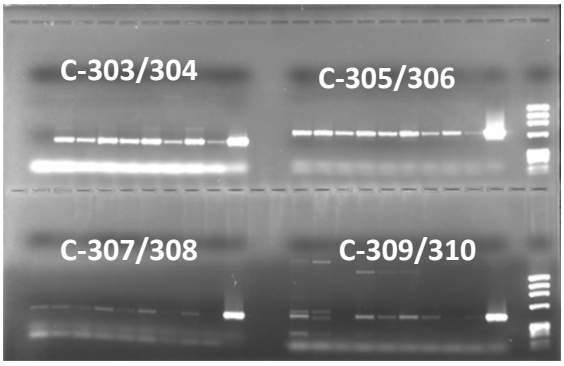

Figure 3 Screening field samples of *Porites lobata* for fragment amplification across individual coral colonies with primer sets designed from cloned *P. lobata Eco*RI restriction fragments. Lane 25, top & bottom MW markers. Gel 0.8% agarose.

Conclusions

Two variations of the AFLP technique were used in an attempt to develop an assay to screen coral for mutation accumulations. The first used the standard AFLP protocol. Results yielded small fragments <500 bp and cloning and sequencing efforts of a subset of DNA fragments showed the presence of DNA from other organisms resident in the holobiont, making this approach unsuitable for directed monitoring of coral mutational events.

The second approach was a modification of the standard AFLP protocol that involved carrying out the procedure using only one restriction enzyme, *Eco*RI with a six-base recognition sequence. This modification did yield fragments approximately 1-1.3 kb. However, subsequent validation procedures yielded only seven coral-like DNA fragments for further analysis. Eight primer combinations of 64 possible combinations were tested. No primer combination tested amplified all samples.

From these experiments, we concluded that the AFLP assay as executed to this point is not suitable for determining mutational changes in coral. Though the modifications implemented did provide a means of tracking coral-specific restriction fragments, in most cases the sequences obtained did not correspond to a particular gene fragment. This eliminated the possibility of determining possible functional

consequences of a mutation. Further, the amount of genome coverage was small, so even if multiple fragments were used in the screening assay it is unlikely there would be sufficient sensitivity to detect even moderate-levels of mutational events over a one to three month timeframe (repeated measures sampling frequency) without further modifications.

Abbreviations:

AP-PCR-Arbitrarily primed polymerase chain reaction
DGGE-Denaturing gradient gel electrophoresis
RAPDs–Random amplification of polymorphic DNA
RFLP-Restriction fragment length polymorphism
SNP-Single-nucleotide polymorphism
SSCP-Single-strand conformation polymorphism
8-oxo-dG - 8-oxo-7,8-dihydro-2'-deoxyguanosine

Table 1. PCR Primers for Coral Genomic Fragments

Primer Designation	Source Species	Source Site	Source Colony	Primer Sequence (5'-3')	Pair with Primer
C303F	*Porites lobata*	Ordnance Reef, Oahu, HI	1-3	AAA GAC GCT CAG TGT TGG GT	C304R
C304R	*Porites lobata*	Ordnance Reef, Oahu, HI	1-3	AAT TGC TGT GCG GCA TTG AA	C303F
C305F	*Porites lobata*	Ordnance Reef, Oahu, HI	1-2	GCC TGA ACA ATG CAA AGC CA	C306R
C306R	*Porites lobata*	Ordnance Reef, Oahu, HI	1-2	GTT GAG CCG GCG ACT AGT AA	C305F
C307F	*Porites lobata*	Maunalua Bay, Oahu, HI	K5	TTC CAT CAT GGT CGT GCA GT	C308R
C308R	*Porites lobata*	Maunalua Bay, Oahu, HI	K5	TAG GTG GGG AAT CAA ACG GC	C307F
C309F	*Porites lobata*	La Perouse, Maui, HI	3-3	GCT GGC TTA CAG GGT AGC AG	C310R
C310R	*Porites lobata*	La Perouse, Maui, HI	3-3	TGC CTA ATG TGG CAC CAA GT	C309F
C311F	*Porites lobata*	La Perouse, Maui, HI	3-3	TAA GGT CTC CCC GAC CGA TT	C312R
C312R	*Porites lobata*	La Perouse, Maui, HI	3-3	ACC AAA AAG AAT CGC CGT GC	C311F
C313F	*Porites lobata*	La Perouse, Maui, HI	3-3	CGT CCG AGA AGT ACG TTC CA	C314R
C314R	*Porites lobata*	La Perouse, Maui, HI	3-3	CTT CTG AGG CTG GTA GGC TG	C313F
C315F	*Porites lobata*	Maunalua Bay, Oahu, HI	K5	TTG CTA TCC CCC AAA CCA CC	C316R
C316R	*Porites lobata*	Maunalua Bay, Oahu, HI	K5	TCT CTT TTT GGG GCG GGA AA	C315F

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